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Relationship between *Helicobacter pylori iceA*, *cagA*, and *vacA* Status and Clinical Outcome: Studies in Four Different Countries

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There is continuing interest in identifying *Helicobacter pylori* virulence factors that might predict the risk for symptomatic clinical outcomes. It has been proposed that *iceA* and *cagA* genes are such markers and can identify patients with peptic ulcers. We compared *H. pylori* isolates from four countries, looking at the *cagA* and *vacA* genotypes, *iceA* alleles, and presentation of the infection. We used PCR to examine *iceA*, *vacA*, and *cagA* status of 424 *H. pylori* isolates obtained from patients with different clinical presentations (peptic ulcer, gastric cancer, and atrophic gastritis). The *H. pylori* isolates examined included 107 strains from Bogota, Colombia, 70 from Houston, Tex., 135 from Seoul, Korea, and 112 from Kyoto, Japan. The predominant genotype differed among countries: the *cagA*-positive *iceA1 vacA* s1c-m1 genotype was predominant in Japan and Korea, the *cagA*-positive *iceA2 vacA* s1b-m1 genotype was predominant in the United States, and the *cagA*-positive *iceA2 vacA* s1a-m1 genotype was predominant in Colombia. There was no association between the *iceA*, *vacA*, or *cagA* status and clinical outcome in patients in the countries studied. *iceA* status shows considerable geographic differences, and neither *iceA* nor combinations of *iceA*, *vacA*, and *cagA* were helpful in predicting the clinical presentation of an *H. pylori* infection.

Helicobacter pylori is the major cause of chronic gastritis and plays an important role in the pathogenesis of peptic ulcer, gastric carcinoma, and primary B-cell gastric lymphoma (7–9, 13, 15). Histological gastritis is essentially universal among *H. pylori*-infected individuals, but only a minority develop a clinically significant outcome, such as peptic ulcer disease or gastric cancer.

Experience with other bacterial pathogens suggests that *H. pylori* strain-specific factors may influence the pathogenicity of different *H. pylori* isolates. *H. pylori* studies have primarily focused on two groups of putative bacterial virulence factors, the *cag* pathogenicity island (for which *cagA* is a marker) and the vacuolating cytotoxin VacA (4, 24). The presence of an intact *cag* pathogenicity island is associated with increased interleukin-8 production and mucosal inflammation (4). Overall, the data support the notion that infection with a *cagA*-positive isolate increases the risk but does not predict the presence of a clinically significant outcome (8, 25, 26). Differences in the *vacA* gene (the mosaic combination of signal [s] regions and middle [m] region allelic types) have been identified, and attempts have been made to associate specific *vacA* genotypes (especially s1-m1 type) with different outcomes, especially with duodenal ulcer (DU) disease (1, 2).

In East Asia, the predominant genotype of the circulating *H. pylori* is *cagA* positive *vacA* genotype s1-m1 irrespective of outcome (10, 11, 14, 19, 23, 28). Recently, a new candidate gene designated *iceA* (for induced by contact with epithelium) was suggested to have an association with peptic ulcer (17, 18). The *iceA* gene has two main allelic variants, *iceA1* and *iceA2*. van Doorn et al. (20) reported that the *iceA* allelic type was independent of the *cagA* and *vacA* status, and there was a significant association between the presence of the *iceA1* allele and peptic ulcer disease. Those researchers proposed that genotyping of *iceA* and *cagA* might offer an effective combination for identification of patients with peptic ulcers. Their results were obtained from patients in The Netherlands, and the search for virulence factors related to outcome of infection has been hampered by the fact that there appear to be differences in the predominant strain in circulation in different geographic regions (6, 12). Thus, conclusions derived from data from a single geographic region may not be true for other geographic regions.

In this study, we examined the *iceA* allelic type in strains from four different countries and its relation with *cagA* status and *vacA* genotypes and clinical outcome.

MATERIALS AND METHODS

Patients and *H. pylori* isolates. We examined 424 *H. pylori* isolates; 107 strains from Bogota, Colombia (46 with gastric cancer, 27 with DU, and 34 with histological gastritis only [gastritis]), 70 from Houston, Tex. (16 with gastric cancer, 28 with DU, and 26 with gastritis), 135 from Seoul, Korea (60 with gastric cancer, 53 with DU, and 22 with gastritis), and 112 from Kyoto, Japan (34 with gastric cancer, 48 with DU, and 30 with gastritis). DUs were identified endoscopically. We excluded the DU cases with gastric ulcer. Gastritis was defined as histological gastritis with no peptic ulcers, gastric cancer, or any esophageal diseases (e.g., gastroesophageal reflux disease and esophageal cancer). Histologically, biopsy specimens were embedded in paraffin, stained with Genta stain (Korea, Colombia, and the United States) or modified Giemsa stain (Japan), and examined in a blind test (the patient's clinical diagnosis and the characteristics of the *H. pylori* strain not known to the individual examining the slide) as described previously (5).

Fifty-nine men and 48 women (mean age, 52.0 years) in Colombia, 48 men and 22 women (mean age, 51.9 years) in the United States, 76 men and 59 women (mean age, 51.8 years) in Korea, and 62 men and 50 women (mean age, 51.8 years) in Japan were studied. For the Korean patients, the mean age of patients with gastric cancer (55.4 years) was significantly higher than that of patients with DU (41.6 years) or gastritis (42.7 years); there were no such age differences for the other groups. No subjects had received treatment for *H. pylori* infection. Informed consent was obtained from all patients, and the protocol was approved by the local ethics committee.

Preparation of *H. pylori* genomic DNA. Gastric biopsy specimens were obtained for isolation of *H. pylori* by previously described culture methods (25, 26,

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TABLE 1. PCR primers for amplification of *cagA*, *vacA*, and *iceA* sequences

Gene and DNA region amplified	Primer	Primer sequence (5′→3′)[a]	Size (bp) of PCR product (location)
cagA	CAGAF	GATAACAGGCAAGCTTTTGAGG	349 (1228–1576[b])
	CAGAR	CTGCAAAAGATTGTTTGGCAGA	
vacA s1	VA1-F	ATGGAAATACAACAAACACAC	259 (797–1055[c])
	VA1-R	CTGCTTGAATGCGCCAAAC	
vacA s2	VA1-F	ATGGAAATACAACAAACACAC	286 (284–569[d])
	VA1-R	CTGCTTGAATGCGCCAAAC	
vacA s1a	S1A-F[e]	TCTYGCTTTAGTAGGAGC	212 (844–1055[c])
vacA s1b	SS3-F[e]	AGCGCCATACCGCAAGAG	187[f]
vacA s1c	S1C-F[e]	CTYGCTTTAGTRGGGYTA	213[f]
vacA m1	VAG-F	CAATCTGTCCAATCAAGCGAG	570 (2071–2640[c])
	VAG-R	GCGTCTAAATAATTCCAAGG	
vacA m2	VAG-F	CAATCTGTCCAATCAAGCGAG	645 (639–1283[d])
	VAG-R	GCGTCTAAATAATTCCAAGG	
iceA1	*iceA*1F	GTGTTTTTAACCAAAGTATC	247 (857–1103[g])
	*iceA*1R	CTATAGCCASTYTCTTTGCA	
iceA2	*iceA*2F	GTTGGGTATATCACAATTTAT	229 or 334[f]
	*iceA*2R	TTRCCCTATTTTCTAGTAGGT	

[a] Y is C or T, M is A or C, S is C or G, and R is A or G.
[b] Nucleotide positions in the *cagA* gene of *H. pylori* ATCC 53726 (GenBank accession no. L117714).
[c] Nucleotide positions in the *vacA* gene of *H. pylori* 60190 (GenBank accession no. U05676).
[d] Nucleotide positions in the *vacA* gene of *H. pylori* Tx30a (GenBank accession no. U29401).
[e] Used with primer VA1-R.
[f] No published coordinates for genes in strains of these types.
[g] Nucleotide positions in the *iceA* gene of *H. pylori* 60190 (GenBank accession no. U43917).

28). All stock cultures were maintained at −80°C in brucella broth (Difco, Detroit, Mich.) supplemented with 20% glycerol (Sigma Chemical Co., St. Louis, Mo.). The strains used in this study were passaged three times on average in each country. *H. pylori* strains were grown at 37°C on brain heart infusion (BHI) (Difco) plates containing 7% horse blood (Cocalico Biological, Inc. Reamstown, Pa.) in a 12% CO_2 incubator with 100% relative humidity. The organisms were identified as *H. pylori* by Gram staining, colony morphology, and positive oxidase, catalase, and urease reactions. Multiple isolates on the plates were pooled together, and genomic DNA was extracted with the QIAamp tissue kit (QIAGEN Inc., Santa Clarita, Calif.) or InstaGene Matrix (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions.

Analysis of *vacA*, *cagA*, and *iceA* by PCR. PCR amplification was performed as previously described (28) for 35 cycles, with 1 cycle consisting of 1 min at 95°C, 1 min at 52°C, and 1 min at 72°C. The final cycle included a 7-min extension step to ensure full extension of the PCR products.

All primers used in this study were presented in Table 1. For detection of the *cagA* gene, primers CAGAF and CAGAR which yield a fragment of 349 bp from the middle conservative region of the *cagA* gene were used.

For analysis of the *vacA* s region, primers VA1-F, VA1-R, SS2-F, and SS3-F described previously by Atherton et al. (1, 2) were used. Primers VA1-F and VA1-R yielded a fragment of 259 bp for s1 variants and a fragment of 286 bp for s2 variants. Each isolate was typed s1b or s2 by performing PCR using primers SS3-F–VA1-R and SS2-F–VA1-R, respectively. Recently, van Doorn et al. (20, 21) found a novel subtype, designated s1c, and all s1c subtype strains were identified as s1a subtype by using the primers described previously by Atherton et al. Therefore, we designed new primers S1A-F and S1C-F specific for the s1a and s1c subtypes, respectively, and each isolate was typed as s1a or s1c by performing PCR with primers S1A-F–VA1-R and S1C-F–VA1-R, respectively.

For analysis of the *vacA* m region, primers VAG-F and VAG-R yielded a fragment of 570 bp for m1 variants and a fragment of 645 bp for m2 variants (3, 28).

For analysis of the *iceA* genotype, primers *iceA*1F, *iceA*1R, *iceA*2F, and *iceA*2R described previously by van Doorn et al. (20) were used. Primers *iceA*1F and *iceA*1R yielded a fragment of 247 bp for the *iceA1* allele, and primers *iceA*2F and *iceA*2R yielded a fragment of 229 or 334 bp according to the existence of repeated sequences of 105 nucleotides.

Data analysis. Fisher's exact test was used for analysis of data for different groups and diseases. A P value of <0.05 was accepted as statistically significant.

RESULTS

***iceA* genotyping.** Overall, *iceA1* was detected in 207 (48.8%) of all 424 isolates examined; *iceA2* was found in 141 isolates (33.3%). Seventy-three isolates (17.2%) were positive for both *iceA1* and *iceA2*, and three isolates (0.7%) did not yield any PCR product for *iceA*.

The *iceA* genotype and clinical outcome were not associated (Table 2). In Japan and Korea, the *iceA1* allele was predominant irrespective of the disease, whereas the *iceA2* allele was predominant irrespective of the disease in the United States. In Colombia, the *iceA2* allele was predominant in gastric cancer and gastritis cases and the prevalence of the *iceA1* allele was equal to that of *iceA2* allele in DU cases; however, these differences were not statistically significant ($P = 0.41$ for gastric cancer versus DU; $P = 0.26$ for gastritis versus DU). The prevalence of isolates with both the *iceA1* and *iceA2* allele was significantly lower in the United States than in Korea, Japan, or Colombia ($P < 0.0001$ for each) (Table 2).

The prevalence of the *iceA1* allele in the four countries was

TABLE 2. *iceA* status of *H. pylori* strains from four countries

Country and clinical outcome	*n*	No. (%) of strains with the following genotype:			No. (%) of strains without *iceA*
		iceA1	*iceA2*	*iceA1 iceA2*	
Korea					
Gastritis	22	17 (77.3)	1 (4.5)	4 (18.2)	0 (0)
Gastric cancer	60	40 (66.7)	9 (15.0)	11 (18.3)	0 (0)
DU	53	37 (69.8)	4 (7.5)	12 (22.6)	0 (0)
Total	135	94 (69.6)	14 (10.4)	27 (20.0)	0 (0)
Japan					
Gastritis	30	17 (56.7)	7 (23.3)	6 (20.0)	0 (0)
Gastric cancer	34	22 (64.7)	6 (17.6)	6 (17.6)	0 (0)
DU	48	30 (62.5)	11 (22.9)	7 (14.6)	0 (0)
Total	112	69 (61.6)	24 (21.4)	19 (17.0)	0 (0)
United States					
Gastritis	26	8 (30.8)	18 (69.2)	0 (0)	0 (0)
Gastric cancer	16	3 (18.8)	12 (75.0)	0 (0)	1 (6.3)
DU	28	0 (0)	25 (89.3)	3 (10.7)	0 (0)
Total	70	11 (15.7)	55 (78.6)	3 (4.3)	1 (1.4)
Colombia					
Gastritis	34	9 (26.5)	17 (50.0)	8 (23.5)	0 (0)
Gastric cancer	46	12 (26.1)	20 (43.5)	12 (26.1)	2 (4.3)
DU	27	12 (44.4)	11 (40.7)	4 (14.8)	0 (0)
Total	107	33 (30.8)	48 (44.9)	24 (22.4)	2 (1.9)

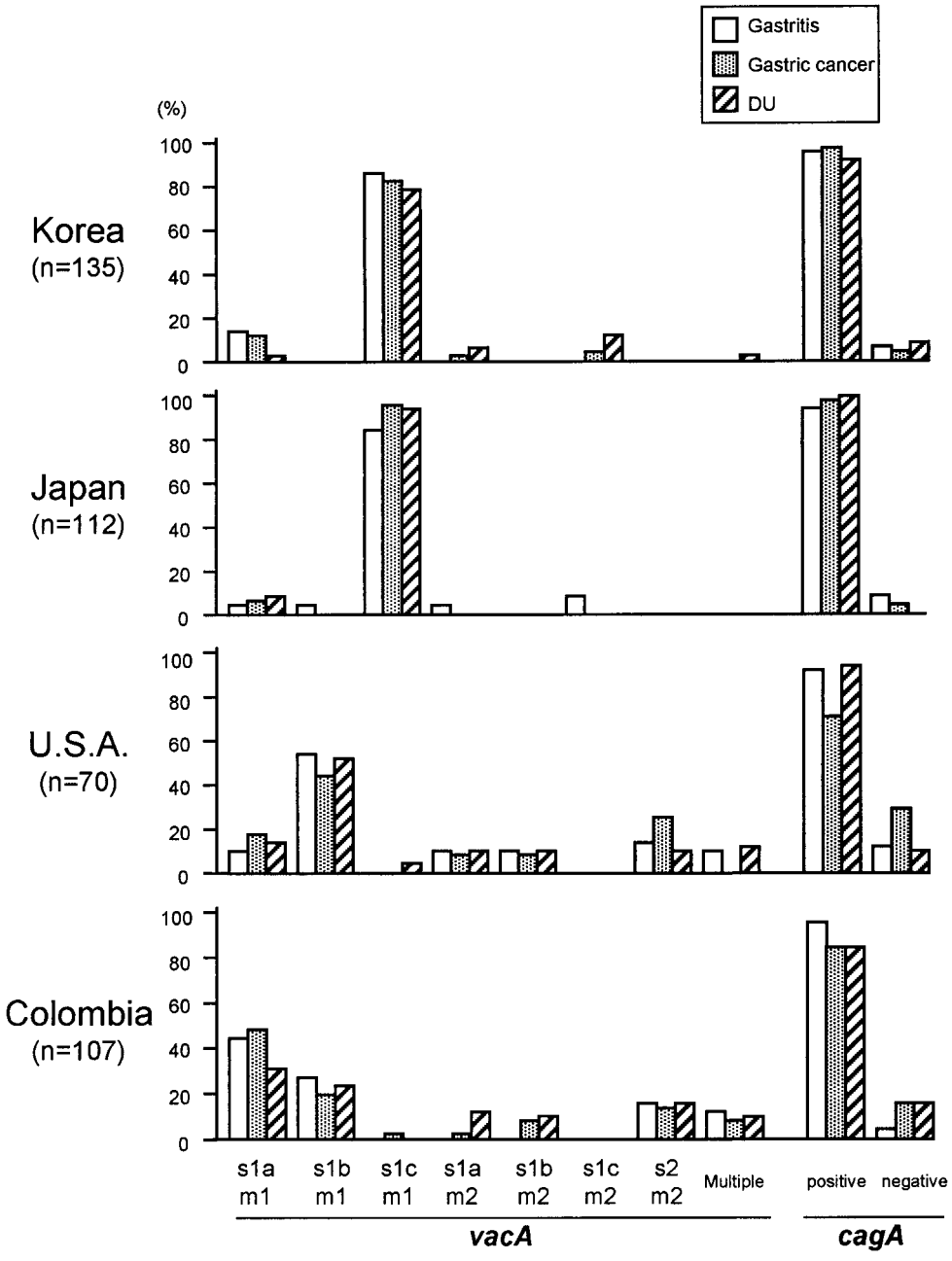

FIG. 1. *vacA* and *cagA* status of *H. pylori* strains from four countries.

assessed among the strains with a single *iceA* allelic type. The prevalence of *iceA1* was significantly higher in Korea and Japan than in the United States and Colombia (Korea or Japan versus the United States or Colombia; $P < 0.0001$ for each) (Table 2). However, in the four countries, there was no association of the *iceA* genotype and either the *cagA* status ($P > 0.6$) or the *vacA* genotype ($P > 0.7$).

As previously reported (20), most isolates with the *iceA2* allele (212 of 214 [99%]) could be divided into two types according to the presence of repeated sequences of 105 nucleotides and whether PCR products were 229 bp (*iceA2*-1) or 334 bp (*iceA2*-2) long. Only two isolates (one Korean gastritis case and one U.S. gastritis case) had the PCR product of about 124 bp, possibly due to the lack of a 105-bp repeat region. Eighteen isolates (8.4%) had both the *iceA2*-1 and *iceA2*-2 alleles.

In Korea, Japan, and the United States, the *iceA2*-1 allele was predominant irrespective of the clinical outcome (data not shown). In Colombia, the *iceA2*-1 allele was predominant in gastritis cases (10 of 17 [59%]) and DU cases (7 of 11 [63%]) and the *iceA2*-2 allele was predominant in gastric cancer cases (14 of 20 [70%]); however, none of these differences were statistically significant ($P < 0.10$).

***vacA* genotyping and *cagA* status.** The *vacA* genotype was significantly different in each country (Fig. 1), precluding an association between *vacA* genotype and clinical outcome. The *vacA* genotype s1c-m1 was predominant in Japan and Korea,

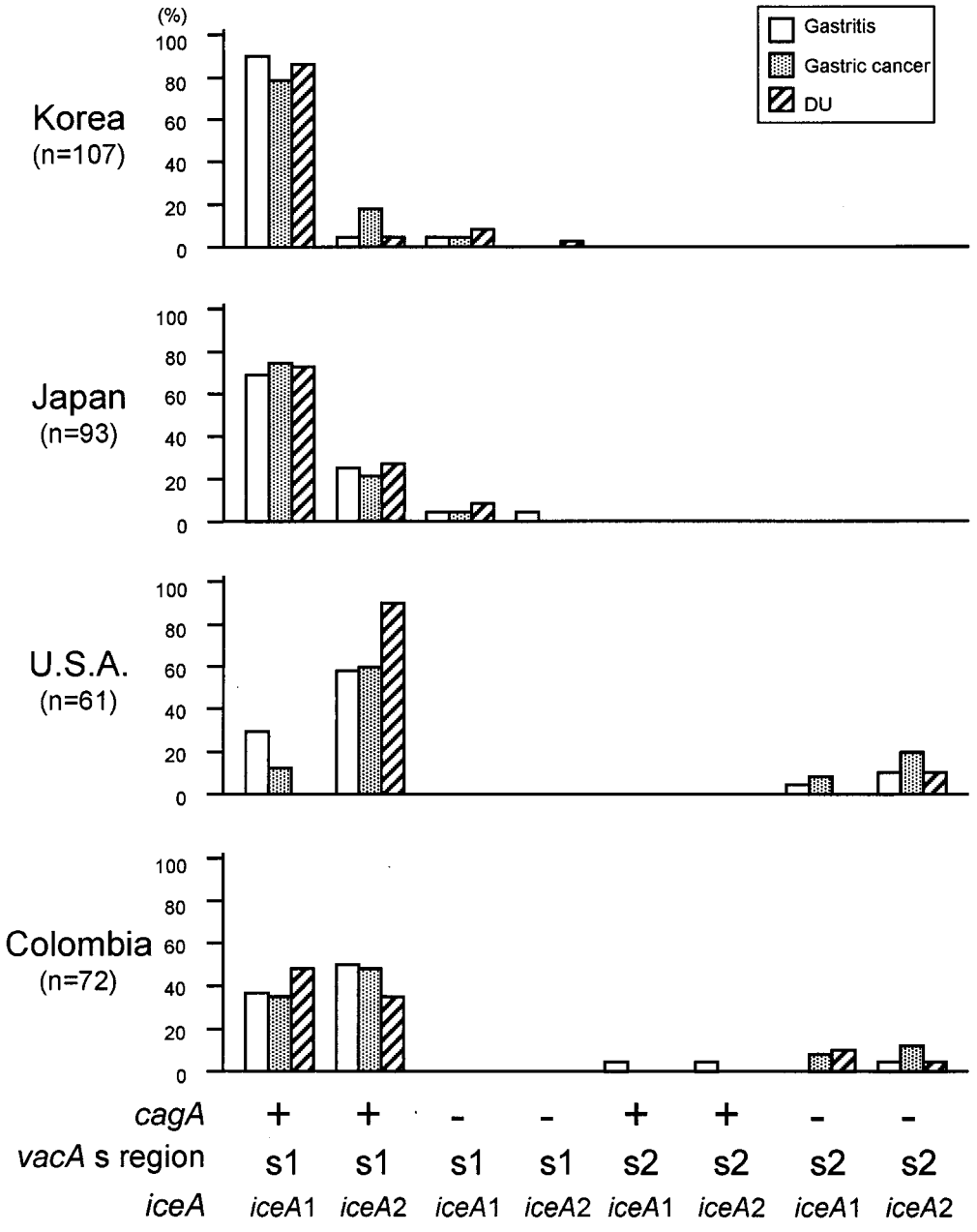

FIG. 2. Combination of *cagA*, *vacA* s region, and *iceA* genotypes and clinical outcome. We examined eight different combinations based on analysis of the *vacA* s region (s1 and s2), *cagA* (positive [+] and negative [−]) and the *iceA* type (*iceA1* and *iceA2*) in patients with a single genotype.

genotype s1b-m1 was predominant in the United States, and genotype s1a-m1 was predominant in Colombia, irrespective of the clinical outcome for patients from each country (Fig. 1).

In this study, *cagA* status was determined by PCR using one set of primers. To avoid false-negative results, *cagA*-negative status was confirmed by immunoblotting in cases yielding no PCR product using *cagA*-specific primers, as previously described (27). As a result, all cases with *cagA* gene-negative results by PCR were also CagA protein negative by immunoblotting. The *cagA* gene-positive isolates were predominant in every country, with no association between *cagA* status and clinical outcome (Fig. 1). The *vacA* genotype s1 was almost always associated with the presence of the *cagA* gene irrespective of the country ($P < 0.0001$ for United States and Colombia). In Japan and Korea, the predominant strain had the *vacA* s1 genotype irrespective of the *cagA* status. For example, of 10 *cagA*-negative strains, 8 had the *vacA* s1c genotype and 2 had the *vacA* s1a genotype.

Combination of *iceA*, *vacA*, and *cagA* genotypes. By using the method of van Doorn et al. (20), we examined eight different combinations based on analysis of the *vacA* s region (s1 and s2), *cagA* (positive and negative), and the *iceA* type (*iceA1* and *iceA2*) in patients with a single genotype (Fig. 2). We were unable to identify an association between these genotypes and clinical outcome. For example, the *cagA*-positive *iceA1 vacA* s1 genotype was predominant in Japan and Korea and the *cagA*-positive *iceA2 vacA* s1 genotype was predominant in the United States irrespective of the clinical outcome.

DISCUSSION

van Doorn et al. (20) examined 94 gastric biopsy specimens from patients in The Netherlands and reported a strong association between the *iceA1* allele and peptic ulcer disease. They also reported that *cagA* positivity and *vacA* s1 genotype were also associated with peptic ulcer disease. Overall, our data are consistent with other recent reports that *cagA* status and *vacA* genotype do not predict clinical outcome (6, 8, 10–12, 14, 19, 23, 25, 26, 28). van Doorn et al. suggested that the addition of *iceA* genotyping might provide a better discrimination. We were unable to confirm an association between the *iceA* allele and clinical outcome. As a general rule, important disease-associated bacterial toxins are tightly associated with their respective diseases and the absence of the factor corresponds with the absence of the diseases in different geographic regions (e.g., cholera toxin and cholera or diphtheria toxin and diphtheria). The fact that predictions based on the *cagA*, *vacA*, or *iceA* genotype were not confirmed in different populations suggests that region-associated observations are possibly being construed as disease-specific associations. This problem continues to plague work on *H. pylori* such that, in the future, it may be prudent to confirm genotypic or phenotypic *H. pylori*-disease associations in several different geographic regions prior to making any claims.

The fact there were geographic differences in both the *vacA* and *iceA* genotypes is interesting. The *iceA*1 allele was predominant in Japan and Korea, and the *iceA*2 allele was predominant in the United States and Colombia. In a study of the geographic distributions of the *vacA* genotype (21), the s1c allele was observed exclusively in isolates from East Asia, which is in agreement with the results of this study. We found that the *vacA* s1a genotype was dominant in Colombia (72 cases). In contrast, a recent report of strains from Central and South America (Brazil, Costa Rica, Peru, and Colombia) suggested that the *vacA* s1b genotype was predominant (22). They evaluated only six Colombian isolates, but the results, if confirmed, suggest that there may be marked variation within broad geographic areas. This is also consistent with the fact that when van Doorn et al. examined 60 U.S. (Nashville, Tenn.) and 13 Canadian strains, they reported that the prevalence of s1a and s1b genotypes was identical (22). In contrast, in Houston, Tex., the s1b strains were predominant. Similar marked differences in the prevalence of *cagA* in Nashville and Houston (1, 2, 6, 12, 16) confirm that regional variations in the dominant circulating strain occur, and failure to take this into account this has repeatedly lead to conclusions that are not true for other geographic regions. It is interesting that although *H. pylori* from Korea and Japan had very similar patterns in *cagA*, *iceA*, and *vacA* status (the *cagA*-positive *iceA1* *vacA* s1c-m1 genotype was predominant), preliminary data suggest that in Taiwan, *vacA* m2 is dominant.

In summary, we were unable to confirm the reports of association of *iceA* status and clinical outcome. *iceA* shows considerable geographic differences, and neither *iceA* nor combinations of *iceA*, *vacA*, and *cagA* were helpful in predicting the clinical presentation of an *H. pylori* infection.

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